Characterisation of polyphenolic compounds in *Clerodendrum petasites* S. Moore and their potential for topical delivery through the skin

Premrutai Thitilertdecha[a,b,*], Richard H Guy[a], Michael G Rowan[a]

[a]Department of Pharmacy & Pharmacology, University of Bath, Bath, BA2 7AY, England.

[b,*]Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2, Prannok Road, Bangkoknoi, Bangkok, 10700, Thailand.

*Tel: +6624198906-8; Fax: +6624198818

E-mail address: premrutai@gmail.com

Abstract

Ethnopharmacological relevance: *Clerodendrum petasites* S. Moore (CP) has been widely prescribed in Thailand and neighbouring countries for both oral and topical administration to treat asthma, fever, cough, vomiting and skin diseases, for at least 30 years.

Aim of the study: To characterise polyphenolic compounds in the plant, to predict the feasibility of their topical absorption and to test their ability to penetrate the skin.

Materials and Methods: Identification and quantification of flavonoids and phenolic acid derivatives in an ethanolic extract of the aerial parts of the plant was carried out using high performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection. Ambiguous isomeric compounds were distinguished by nuclear magnetic resonance (NMR) spectroscopy. The feasibility of the compounds' topical permeability was evaluated by predicting their maximum fluxes from their physicochemical properties. The skin penetration of compounds in the plant extract was measured *in vitro* over 24 hours.

Results: Vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin and chrysin, were identified in CP. All compounds except apigenin and hispidulin are reported in this species for the first time. Hispidulin is the predominant compound (1.2% w/w in a dried ethanolic extract) followed by nepetin, verbascoside, vanillic acid, and apigenin. Across mammalian skin, hispidulin was percutaneously absorbed within 3 hours and vanillic acid and nepetin permeated the skin after 6 hours. These experimental observations were consistent with the predicted maximum fluxes of these compounds calculated from their physicochemical properties.

Conclusions: Many of the phenolic compounds reported in this study are well known to possess antimicrobial, anti-inflammatory and anti-oxidant activities. The skin permeation studies reported here support traditional topical uses of the plant in skin treatments and are useful for further topical formulation optimisation.

Abbreviations

CP, *Clerodendrum petasites* S. Moore; HPLC, high performance liquid chromatography; PDA, photodiode array; UV, ultraviolet; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.

Chemical compounds studied in this article

Vanillic acid (PubChem CID: 8468); verbascoside (PubChem CID: 5281800); nepetin (PubChem CID: 5317284); hispidulin (PubChem CID: 5281628).

Keywords

Clerodendrum petasites; Thai traditional medicine; HPLC-MS; topical delivery; phenolic compounds

1. Introduction

Clerodendrum petasites (English name: One Root Plant) is one of ~700 species of this genus in the family Lamiaceae (*Clerodendrum petasites* S. Moore, 2005; The plant list, 2010). The plant is widespread in the middle, north-eastern, and southern parts of Thailand. There are numerous Thai names from each region, for instance, Ping-Khom and Ping-Luang in the north, Phaya-Rak-Deaw in the south, Nang-Shon and Phom-Phee in the northeast. However, Thao-Yaai-Mom from the midlands is the best known.

Thai traditional practitioners usually prepare aerial parts, leaves, or roots of *C. petasites* as a tea, alcoholic extract or cigarette to treat asthma (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003; Panthong *et al.*, 1986). Leaves and roots are also ground into powders for treatment of inflammation (Panthong *et al.*, 1986) as well as to treat fever, cough, and vomiting (Panthong *et al.*, 2003; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007) (S. Tungjitaruen, pers. comm., 2011). The plant is widely prescribed for oral administration and generally formulated into multi-herb recipes. The most famous recipe is “Ha-Rak” (synonyms: Ben-Cha-Lo-Ka-Wi-Chian, Kaew-Ha-Dueng, Phed-Sa-Wang), containing equal amounts by weight of five roots from *C. petasites*, *Ficus racemosa* Linn, *Capparis micracantha* DC, *Harrisonia perforate* Merr, and *Tiliacora triandra* Diels (Pichaensoonthon *et al.*, 2005). The recipe is currently registered by the Thai Food and Drug Administration (FDA) for antipyretic activity (List of herbal medicinal products, 2006; National list of essential medicines: Ha-Rak). Dosage forms of Ha-Rak are powders, tablets and capsules, but decoction is conventionally served. There are fewer records for topical remedies. Poultices are most often formulated for skin diseases, such as rash, abscess, urticaria, snakebites and insect bites (Panthong *et al.*, 2003; Pongboonrot, 1965; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007) (T. Tipcharoentham, pers. comm., 2011; S. Tungjitaruen, pers. comm., 2011). Many recipes are dispersed in alcohol, especially Thai rice whisky, before application.

C. petasites is also widely distributed in many other countries, e.g., Malaysia, India, Southern China, Sri Lanka, and Vietnam. Ethnomedical uses of the plant are found in their medical systems. For example, root and leaf extracts of *C. petasites* have been documented for the treatment of rheumatism, asthma and other inflammatory diseases (Shrivastava and Patel, 2007). In India, fruits are reportedly used to reduce fertility in males and the plant is used to cure malaria in China (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003; Shrivastava and Patel, 2007).

Although the chemical constituents in the genus *Clerodendrum* have been widely investigated, there have been only a few studies on *C. petasites*. The compounds previously reported in the aerial parts and roots of *C. petasites* include apigenin, hispidulin, 6,4’-dimethoxyscutellarin, hispidulin 7-methylglucuronide, nevadensin 7-glucoside, arbutin and bungene A (Hazekamp *et al.*, 2001; Klaiklay, 2009; Singharachai *et al.*, 2011; Thongchai *et al.*, 2007). There have been no clinical trials that identify and verify the compounds that elicit useful pharmacological effects following topical delivery. Thus, in this study, flavonoids and other phenolic compounds, which are well-known as strong antioxidants with free radical scavenging and metal chelating activities (Perron and Brumaghim, 2009; Robak and Gryglewski, 1996; Wuguo *et al.*, 1997), and are extensively used in dermatological and cosmetological applications (Arct *et al.*, 2002; Arct and Pytkowska, 2008; Bonina *et al.*, 1996; Cimino and Saija, 2005; Lin *et al.*, 2008), were characterised and their topical absorption determined using a pig skin model. Experimental values were compared with theoretical transdermal fluxes calculated from the physicochemical properties of the compounds (Potts and Guy, 1992).

2. Materials and methods

2.1 Plant materials

Dried samples of the aerial parts of *C. petasites* were authenticated by macroscopic identification and obtained from the Ayurved Siriraj Manufacturing Unit of Herbal Medicines and Products, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Extracts were produced by maceration using 80% ethanol and subsequently evaporated to dryness. Five batches of ethanolic extracts were kept separately in light protective and airtight containers and stored in a desiccator at room temperature.

The ethanolic extracts were separated into water, butan-1-ol, ethyl acetate and petroleum ether soluble fractions by liquid-liquid partition. Only the butanol and ethyl acetate fractions were further separated by column chromatography using a step gradient of 100% ethyl acetate followed by 1%, 2%, 5%, 10%, 20%, 50% methanol in ethyl acetate and 100% methanol. All the fractions were kept in light protective and airtight containers and stored at 4°C. The fractions were subsequently examined by NMR to elucidate the structure of ambiguous isomers.

2.2 Chemicals and reagents

Caffeic acid, 4-coumaric acid, naringin, chrysin, 5,7-dimethoxycoumarin, gallic acid, rosemarinic acid, kaempferol, cinnamic acid (Sigma-Aldrich, USA), vanillic acid, ferulic acid, apigenin (Fluka Analytical, China), rutin, quercetin (Koch-Light Laboratories Ltd., UK), verbascoside, naringenin, chrysoeriol, hesperetin, luteolin, diosmetin, nepetin, scutellarein (Extrasynthese, France), hispidulin (Tocris Bioscience, UK), cirsimaritin (BioBioPha. Co; Ltd.), were of analytical grade.

Mobile phases for HPLC-MS and HPLC-PDA consisted of HPLC grade acetonitrile (Fisher Scientific, UK), HPLC grade water obtained from a deionized water treatment system (Milli-pore, MA, USA) and MS grade acetic acid (Fluka Analytical, Germany). Deuterated-methanol (methanol-D_4, CD_3OD), deuterated-chloroform (chloroform-D, $CDCl_3$) and deuterium oxide (D_2O) were used for NMR analysis and purchased from Cambridge Isotope Laboratories, Inc., UK. Other chemicals and reagents, methanol, ethanol (Sigma-Aldrich, USA), butan-1-ol (Fisher Scientific, UK), ethyl acetate, and petroleum ether, tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Acros Organics, USA), tris aminomethane (Trizma® base, Sigma-Aldrich, USA), were of analytical grade.

Excipients of the preliminary topical formulations comprised propylene glycol (Acros Organics, UK) and Vaseline white (Riedel-de Haën, Germany).

2.3 Skin

Fresh porcine abdominal skin was obtained from B&J Pigs Ltd, Somerset, UK. Excessive hair was carefully trimmed using scissors. After cleaning with running cold water, the skin was dermatomed (Zimmer electric dermatome, Oklahoma, USA) to a nominal thickness of 750 µm. The dermatomed skin was sealed in a plastic bag and stored at -20°C until use.

2.4 Preparation of standard solutions

Stock solutions (0.1 mg·mL^{-1}) of the phenolic standards were prepared by dissolution in methanol followed by sonication for 30 minutes where necessary (Fisherbrand® FB11002, Thermo Fisher Scientific Inc., UK). Each analyte stock solution was diluted with methanol to appropriate concentrations for the establishment of calibration curves and validation tests. All standard solutions were filtered through a 0.45 µm nylon membrane (Chronus® filter, LabHut Ltd., UK) before HPLC-MS or HPLC-PDA analysis. Both stock and diluted solutions were stored at 4°C.

2.5 Preparation of plant sample solutions

The dried extract of *C. petasites* was accurately weighed and dissolved in methanol at a concentration of 50 mg·mL^{-1} and sonicated for 30 minutes. After centrifugation at 4000 rpm for 20 minutes (U-32, Boeco, Germany), the supernatant was filtered through a 0.45 µm nylon membrane and diluted with methanol to appropriate concentrations prior to HPLC-MS or HPLC-PDA analysis. The filtered plant sample solution was stored at 4°C.

2.6 Preparation of topical formulations

A solution containing the CP extract at a concentration of 50 mg·mL^{-1} in 50% aqueous ethanol was prepared. The solution was sonicated for 60 minutes and then centrifuged at 4000 rpm for 20 minutes. The supernatant was filtered through a 0.45 µm nylon membrane before use in the *in vitro* permeation tests. The solution was used within 24 hours of preparation.

A paste consisting of 50% CP, 17% propylene glycol and 33% Vaseline (w/w) was prepared and well mixed. The paste was used within 24 hours of preparation.

2.7 HPLC-MS

Experiments were performed on a Shimadzu HPLC-2010A HT system (Shimadzu Corp., Kyoto, Japan) consisting of an autosampler, vacuum degasser, and UV detector which was set at the detection wavelengths of 260 and 330 nm (chosen on the basis of HPLC-PDA results of individual standards).

The HPLC was connected to a Shimadzu MS-2010EV system (Shimadzu Corp., Kyoto, Japan) with a dual source of electrospray ionization and atmospheric pressure chemical ionization (ESI/APCI, DUIS-2010, Japan). Ionization was achieved in both negative- and positive-ion-modes with detector voltage set at 1.5 kV. Nitrogen was used as the nebulising gas, heated to 480°C and delivered at a flow rate of 1.5 L·min^{-1}. MS signals were collected in the scan mode between 50-1000 m/z for identification of chemical components and the single ion-monitoring (SIM) mode was used for quantification of individual compounds.

The column used was a Dionex Acclaim® 120 (C18, 5 µm, 150 x 4.6 mm i.d.). A combination of acetonitrile (A) and 0.1% aqueous acetic acid (v/v, B) was used as mobile phase with an optimized gradient system of 20% A, 80% B for 9 min, 20-60% A, 80-40% B for 6 min, 60% A, 40% B for 5 min, 60-95% A, 40-5% B for 10 min, 95% A, 5% B for 5 min and 20% A, 80% B for 25 min. The injection volume was 20 µL and the flow rate was 0.5 mL·min^{-1}. The column temperature was maintained at 35°C throughout the analysis. All data acquired were processed by the LabSolutions LCMS Software (Shimadzu Corp., Kyoto, Japan).

2.8 HPLC-PDA

The HPLC-PDA system comprised an ASI-100 automated sample injector, thermostatted column compartment TCC-100 and PDA-100 photodiode array detector (Dionex® Ltd., UK). The UV detection wavelengths were set at 260 and 330 nm for quantification and the maximum wavelengths (λ_{max}) of each peak were detected by a wavelength scan from 240 to 360 nm for peak confirmation.

A HiQ Sil C18 HS column (C18, 5 µm, 150 x 4.6 mm i.d., Kyatech, Japan) was used and the temperature was maintained at 35°C. The HPLC-PDA conditions were slightly changed from those which had been optimized for HPLC-MS. Acetonitrile (A) and a mixture of 0.1% aqueous acetic acid and acetonitrile (v/v, 80:20, B) were combined as the mobile phase in a gradient system of 0% A, 100% B for 9 min, 0-50% A, 100-50% B for 6 min, 50% A, 50% B for 5 min, 50-94% A, 50-6% B for 10 min, 94% A, 6% B for 5 min and 0% A, 100% B for 25 min with a flow rate of 0.5 mL·min^{-1}. 20 µL of each sample was injected. Chromatograms were interpreted with Chromeleon software (Dionex®

Ltd., UK). Retention times (t_R) and UV peak detection using HPLC-PDA were compared with those using HPLC-MS.

2.9 Nuclear magnetic resonance spectroscopy (NMR)

All ethanolic extracts, solvent partition fractions, and phenolic standards were dissolved in appropriate solvents (e.g., deuterated-methanol (CD_3OD), deuterated-chloroform ($CDCl_3$) and deuterium oxide (D_2O)). ^1H NMR (500 MHz) spectra were obtained on a Varian Mercury spectrometer. Chemical shifts (δ) were recorded in parts per million (ppm).

2.10 Prediction of maximum flux (J_{max})

A maximum possible flux (J_{max}, $\mu g \cdot cm^{-2} \cdot h^{-1}$) of transport of each compound was calculated from an algorithm derived from Fick's first law of diffusion as follows:

$$J_{max} = k_p \cdot C_{sat,W} \qquad \text{Eq. 1}$$

where k_p is the compound's permeability coefficient ($cm \cdot h^{-1}$) and $C_{sat,W}$ is the saturation solubility of the compound in water ($\mu g \cdot cm^{-3}$). The k_p value is estimated by the Potts and Guy equation (Eq. 2) (Potts and Guy, 1992).

$$\log k_p = -2.72 + 0.71 \cdot \log P - 0.0061 \cdot MW \qquad \text{Eq. 2}$$

where P is the compound's octanol-water partition coefficient and MW is its molecular weight (Da).

However, because the viable epidermis can represent a significant barrier to the penetration of lipophilic compounds, the Potts and Guy estimated k_p (which assumes the transport across the skin is controlled uniquely by the SC) is corrected as proposed by Cleek and Bunge (1993) as follows:

$$k_p^{corr} = \frac{k_p}{1 + \frac{k_p \cdot \sqrt{MW}}{2.6}} \qquad \text{Eq. 3}$$

It follows that J_{max} for the putative active species in the plant extracts can be predicted from Eqs. 1-3 using available or calculable values of MW, log P and $C_{sat,W}$ (ALOGPS 2.1 algorithm, 2001; Chemspider).

2.11 *In vitro* skin permeation

The skin permeation of compounds in the plant extracts was determined using vertical, glass Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA). The exposed membrane surface area was 1.77 cm^2 and the receptor volume was 7.5 mL. The receptor solution was a mixture of ethanol and 5 mM Tris buffer in ratio of 1:4 v/v, at pH 7.3 (slightly less than 7.4 due to the presence of ethanol). Frozen dermatomed pig abdominal skin was thawed for 30 minutes before use and examined visually for punctures or defects. The skin was stripped with one adhesive tape (3.5 cm x 3.5 cm, Scotch book tape, 3M, MN, USA) to remove SC disjunctum before being mounted into the Franz cell. After temperature equilibration at 37°C, the formulations were applied to the skin surface and occluded with Parafilm™ (Bemis®, USA). The amounts of drug applied were 1 mL for the CP solution and approximately 0.2 g for the CP paste. Samples were withdrawn at 3, 6, and 24 hours. At each sampling time, the whole receptor solution volume was removed and replaced with fresh buffer. The samples were stored at 4°C under light protection before quantitative analysis. Six replicates were performed with each formulation.

2.12 Validation and statistical analysis

2.12.1 Limits of detection and quantification (LOD and LOQ)

Each standard solution was diluted and measured in triplicate to assess a signal-to-noise ratio (S/N). The S/N was the ratio of the height of the chromatographic signal above the baseline and the height of the baseline noise measured more than 30 seconds before and after the peak to avoid any peak tails. The concentration with S/N ≥ 3 was defined as LOD and that with S/N ≥ 10 was identified as LOQ.

2.12.2 Calibration curves

Separate calibrations were carried out for HPLC-MS and HPLC-PDA assays. At least six concentrations and three independent preparations of phenolic standards in the range of 0.25 – 20 ng in methanol were injected into the HPLC-MS detector. 0.25 ng – 2 µg of standard mixtures in methanol were subjected to HPLC-PDA detection. Calibration curves were obtained by plotting the areas under the curves (AUCs) against concentration and the equation of the line determined by linear regression. The curves were used only within the linear range.

2.12.3 Precision

Three different concentrations (low, middle, and high examples on the calibration curves) of individual phenolic standards were measured five times a day to determine intra-day variability. The standards were also analysed twice a day on three consecutive days in order to obtain inter-day variability. The results were expressed in terms of relative standard deviation (RSD).

2.12.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 5 (GraphPad Software Inc., CA, USA). Calibration curves were analysed with linear regression. Datasets were expressed as mean ± SD (standard deviation) and compared for statistical significance at $P \leq 0.05$ with two-way ANOVA and Bonferroni post-tests.

3. Results and discussion

3.1 Analytical method optimisation and validations of phenolic standards

Twenty four phenolic standards were selected for preliminary qualitative analysis and detected by the optimised HPLC-MS and HPLC-PDA (Table 1).

Table 1. Retention times (t_R), mass to charge ratios (m/z), ion modes of MS detection, and maximum wavelengths (λ_{max}) of phenolic standards from HPLC-MS and HPLC-PDA analyses.

Compound	HPLC-MS			HPLC-PDA	
	t_R (min)	m/z	Ion mode	t_R (min)	λ_{max} (nm)
Gallic acid	4.6	169	(-)ve	5.0	272
Caffeic acid	7.6	179	(-)ve	9.0	324
Vanillic acid	7.8	167	(-)ve	9.9	261
Rutin	9.1	609	(-)ve	11.8	257
Verbascoside	9.9	623	(-)ve	12.7	330
4-Coumaric acid	11.5	163	(-)ve	15.3	310
Ferulic acid	13.9	193	(-)ve	16.4	322
Rosemarinic acid	15.1	359	(-)ve	16.8	329
Naringin	16.2	579	(-)ve	16.3	284
Scutellarein	17.5	287	(+)ve	17.9	334
Luteolin	18.3	287	(+)ve	18.6	347
Nepetin	18.4	315	(-)ve	18.7	345
Quercetin	18.6	301	(-)ve	18.8	256
Cinnamic acid	19.3	147	(-)ve	19.6	282
Apigenin	19.3	271	(+)ve	19.8	334
Naringenin	19.4	271	(-)ve	19.7	291
Hispidulin	19.4	301	(+)ve	20.0	334
Kaempferol	19.5	287	(+)ve	20.0	out of scanning range
Chrysoeriol	19.6	299	(-)ve	20.1	345
Diosmetin	19.6	301	(+)ve	20.1	344
Hesperetin	19.8	301	(-)ve	20.1	289
Cirsimaritin	21.1	315	(+)ve	22.1	333
5,7-Dimethoxycoumarin	21.6	207	(+)ve	22.2	327
Chrysin	22.9	253	(-)ve	24.0	268

From preliminary MS spectra of the plant samples, eleven phenolic compounds were tentatively identified and selected as characteristic markers. Vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, nepetin, naringenin, hesperetin and chrysin were detected in the negative ion mode as [M-H^-] ions, whereas luteolin, apigenin, and hispidulin were detected in the positive ion mode as [M+H^+] ions.

The linearity of the concentration versus peak area relationships was determined over the range of 0.01-6 µM. Linear correlations (r^2) were obtained with $r^2 > 0.95$ for all the eleven phenolic standards except hesperetin ($r^2 = 0.90$). Based on a 20-µL injection, the limits of detection (LOD) and quantification (LOQ) for each standard were determined to be 20-25 nM and 41-50 nM, respectively for luteolin, apigenin, naringenin, hispidulin, hesperetin and chrysin. LODs of vanillic acid, 4-coumaric acid and ferulic acid were 32-38 nM and those of verbascoside and nepetin were 60 and 79 nM, respectively; the LOQs of these compounds were in the range of 63-80 nM except for nepetin (158 nM).

Multiple injections were carried out to determine the precision of the assay for each standard. The intra-day RSD (relative standard deviation) values at medium and high concentrations of each of the standard calibration curves were less than 5% for the eleven phenolic standards except for 4-coumaric acid (6.3%), while the intra-day RSDs at low concentration were relatively higher (less than 9% for the eight phenolic compounds, 13% for 4-coumaric acid and hispidulin, and 19% for ferulic acid). The inter-day RSD values were not significantly different among the low, medium and high

concentrations (below 10% for all compounds except 4-coumaric acid, luteolin, apigenin, and hesperetin for which the RSD was less than 18.5%).

3.2 Identification of chemical compounds in CP

To characterise naturally-occurring chemical compounds in CP (Table 2), three independent criteria corresponding with pure standards were routinely applied: (a) retention time (t_R), (b) mass to charge ratio (m/z), (c) maximum wavelength (λ_{max}). Peaks with a retention time identical to one of the standards and one other criterion in common with that standard were regarded as tentatively identified in this study; peaks with two other criteria in common with the standard were regarded as identified.

Table 2. The characteristic peaks of ethanolic extracts of *C. petasites* (0.1 mg·mL^{-1} in methanol) with MS detection in both negative and positive ion modes. Three independent criteria corresponding with pure standards: (a) retention time (t_R), (b) mass to charge ratio (m/z), and (c) maximum wavelength (λ_{max}), were applied.

R_t (min)	m/z (-)ve	m/z (+)ve	λ_{max} (nm)	Identification	Type of identification
6.2	-	476	-	Unknown	-
7.4	179	-	-	Unknown	-
7.8	-	564	-	Unknown	-
8.0	167	-	261	Vanillic acid	a, b, c
10.1	623	-	330	Verbascoside	a, b, c
11.9	163	-	-	4-Coumaric acid	a, b
14.1	193	-	322	Ferulic acid	a, b, c
18.4	-	287	-	Luteolin	a, b
18.5	315	317	345	Nepetin	a, b, c
19.3	-	271	333	Apigenin	a, b, c
19.4	271	-	-	Naringenin	a, b
19.6	299	301	334	Hispidulin	a, b, c
19.8	301	-	-	Hesperetin	a, b
20.5	-	297	-	Unknown	-
23.0	253	-	-	Chrysin	a, b
23.8	-	315	-	Unknown	-
25.8	-	415	-	Unknown	-
26.8	-	505	-	Unknown	-
34.5	-	458	-	Unknown	-

Eleven compounds of CP were characterised as vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, luteolin, nepetin, naringenin, hispidulin, hesperetin, and chrysin. However, using the above criteria, hispidulin could not be reliably separated from the two isomeric forms: chrysoeriol and diosmetin. They share the same MW, polarity and chromophores, and thus they cannot be unambiguously identified by either MS or UV detection. ^{1}HNMR analysis of an enriched fraction of the extract obtained by column chromatography was used to elucidate which of these isomers was present. Fig. 1 shows that peaks observed in the enriched fraction matched those of hispidulin. No matching NMR peaks for chrysoeriol and diosmetin were observed in the enriched extract (Fig. 2). Thus, the molecule with MW 300 Da in CP is confidently confirmed as hispidulin by four independent criteria of identification. Although hispidulin has been previously reported (Hazekamp *et al.*, 2001; Klaiklay, 2009; Singharachai *et al.*, 2011), none of this earlier work has unambiguously excluded the isomers by NMR spectroscopy.

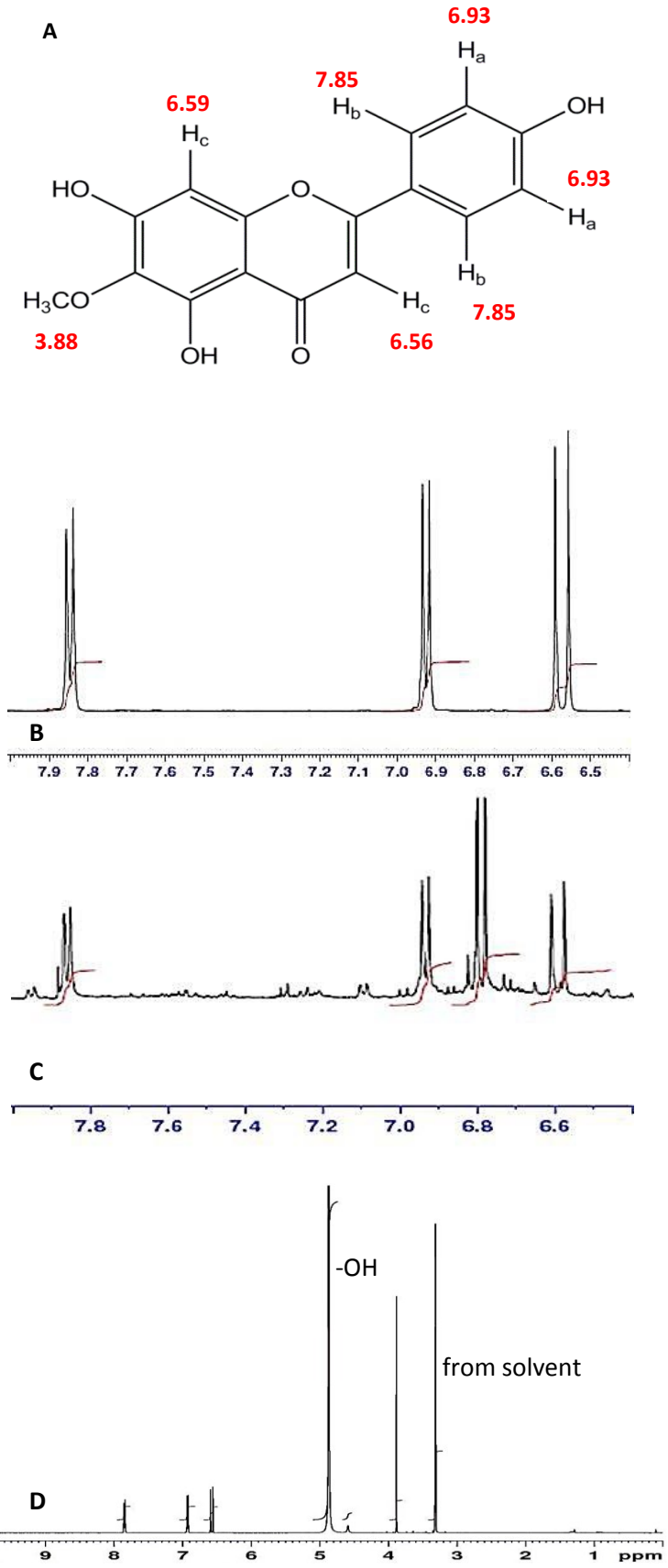

Fig. 1. NMR spectra (^{1}H, 500 MHz in CD_3OD) of *C. petasites* extract and that of a hispidulin standard: (A) chemical structure of hispidulin, (B) NMR spectrum of hispidulin standard (aromatic region), (C) NMR spectrum (range 6-8 ppm) of an enriched fraction from the *C. petasites*, (D) full NMR spectrum (range 0-10 ppm) of hispidulin.

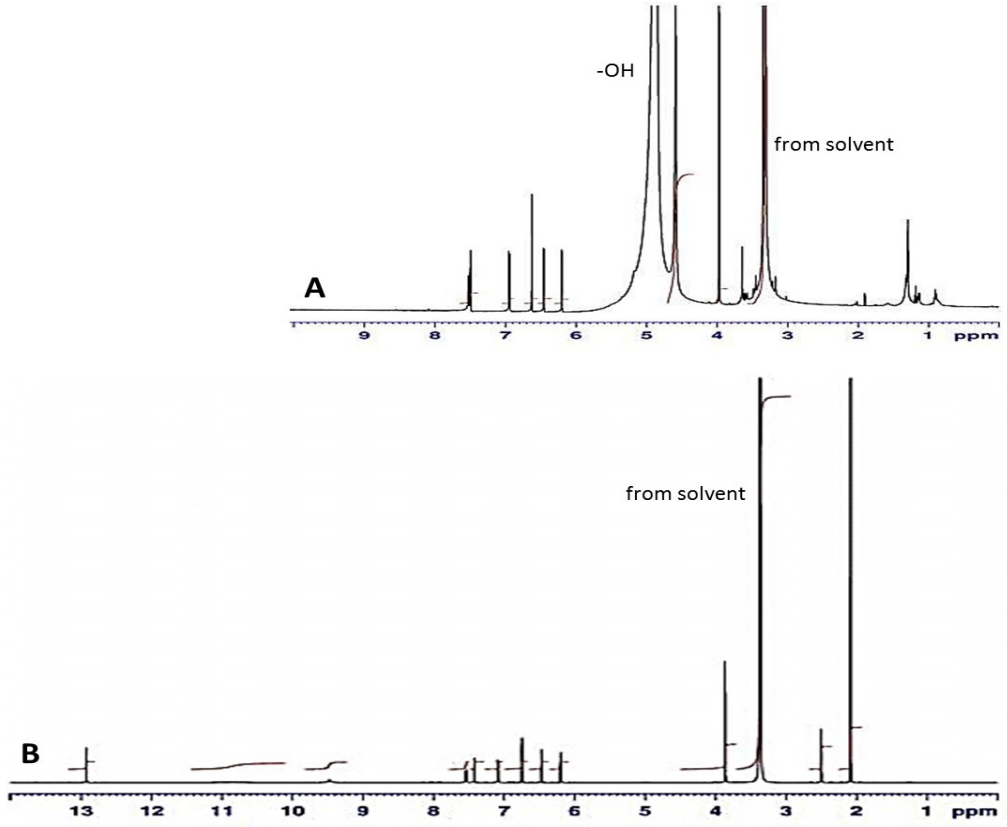

Fig. 2. NMR spectra (^{1}H, 500 MHz in CD_3OD) of (A) chrysoeriol and (B) diosmetin standards in the ranges of 0-10 ppm and 0-14 ppm, respectively.

3.3 Quantification of natural constituents in CP

Vanillic acid, verbascoside, nepetin, apigenin and hispidulin were quantified in the ethanolic extracts (Table 3). The amounts of the other six compounds, 4-coumaric acid, ferulic acid, luteolin, naringenin, hesperetin and chrysin, fell below their LOQs. Hispidulin was predominant at 39 µmol/g followed by nepetin (15 µmol/g), verbascoside (4 µmol/g), vanillic acid (3 µmol/g) and apigenin (1 µmol/g), respectively. Reproducibility of the ethanolic extraction among five different batches was good.

Table 3. Amounts of phenolic constituents in *C. petasites* from five extracts with MS detection in both negative and positive ion modes.

	Amount (µmol/g) in dried extract of *C. petasites*				
Batch no.	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Apigenin**	**Hispidulin**
1	3.4	4.8	17.9	0.7	35.7
2	2.5	3.9	12.8	-	29.8
3	3.7	5.3	17.4	1.2	49.3
4	2.0	2.9	11.5	0.6	35.0
5	2.0	2.5	12.8	1.0	42.5
Average ± SD	*2.7 ± 0.8*	*3.9 ± 1.2*	*14.5 ± 2.9*	*0.9 ± 0.3*	*38.5 ± 7.6*

3.4 Prediction of maximum fluxes (J_{max}) of the phenolic reference compounds

The feasibility of skin absorption of the eleven naturally-occurring phenolic compounds was evaluated by predicting their maximum fluxes (J_{max}) (Table 4) from their physicochemical properties: molecular weight (MW), octanol-water partition coefficient (log P), and water solubility ($C_{sat,w}$) (ALOGPS 2.1 algorithm, 2001; Chemspider).

Table 4. Physicochemical properties (molecular weight (MW), octanol-water partition coefficient (log P), and water solubility ($C_{sat,w}$)) of the eleven phenolic compounds and their predicted maximum permeation rate (J_{max}).

Compound	MW (Da)	log P	$C_{sat,w}$ [b] (mM)	Predicted J_{max} (nmol·cm^{-2}·h^{-1})
Vanillic acid	168.2	1.4[a]	38.0	73.3
Verbascoside	624.6	-0.03 ± 1.0[b]	1.5	0.0005
4-Coumaric acid	164.2	1.5[a]	11.0	23.5
Ferulic acid	194.2	1.5[a]	9.5	14.6
Nepetin	316.3	2.0 ± 0.8[b]	0.4	0.2
Luteolin	286.2	2.5[a]	0.6	1.3
Apigenin	270.2	2.3 ± 0.6[b]	0.8	1.5
Naringenin	272.3	2.5[a]	1.1	3.0
Hispidulin	300.3	2.2 ± 0.7[b]	0.4	0.4
Hesperetin	302.3	2.1[a]	0.8	0.7
Chrysin	254.2	3.5[a]	0.5	8.0

a = experimental value; b = calculated value

The eleven compounds may be broadly categorised into three groups: phenolic acids, flavonoid aglycones, and a phenolic glycoside. Phenolic acids including vanillic acid, 4-coumaric acid and ferulic acid, have the highest predicted J_{max} because of their smaller size and greater water solubility. Flavonoid aglycones, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin, and chrysin, have slightly larger MW and are less soluble in water than the phenolic acids; hence, their predicted penetration rates are slower. Verbascoside is the only phenolic glycoside in this study and contains two sugars, rhamnose and β-glucose; it has the highest MW as a result and a lower log P which (despite its reasonable water solubility) means that this compound has the lowest predicted J_{max}.

Overall, ten of the eleven compounds have predicted fluxes of at least ~0.1 μg·cm^{-2}·h^{-1}; that of verbascoside is so small that it seems very unlikely to result in any measurable therapeutic effect. The phenolic acids have physicochemical properties consistent with a reasonable skin permeability, i.e., modest MW and log P in the range of 1-3 (Hadgraft and Guy, 2003).

3.5 *In vitro* percutaneous absorption

To identify which of the naturally-occurring compounds in CP are able to penetrate through the skin and to evaluate their permeability, *in vitro* experiments using a pig skin model with simple formulations (50% w/w CP paste and a 50 mg·mL^{-1} CP solution in 50:50 v/v ethanol:water) were performed over a 24 hour period. Both formulations represented models for Thai traditional preparations based on natural oils and alcohol. From a pharmaceutical view point, a paste can contain the highest amount of a powdered plant (up to 50% of the total recipe) whereas a solution is the dosage form that might be manipulated easily to achieve the maximum fluxes of the ingredients. Propylene glycol in the paste was used to disperse the powdered plant, to facilitate dissolution of hydrophobic ingredients and to increase skin penetration. 50% aqueous ethanol in the solution also acted as a powerful cosolvent and potential skin penetration enhancer; in addition, it has been reported to be an acceptable donor vehicle for *in vitro* diffusion experiments. Samples were withdrawn from the receptor solution at 3, 6 and 24 hours. Control experiments, in which no formulations were applied, revealed no penetration of any of the compounds present in CP.

Hispidulin was the only compound that penetrated from both formulations through the skin in an amount that could be quantified after 3 hours (Table 5). While both vanillic acid and nepetin applied as a CP solution were detectable in the receptor phase at 3 hours, they could not be quantified

because of their relatively high LOQs (74 and 158 mM, respectively) compared to that of hispidulin (42 mM). Verbascoside delivered from the CP paste was not detected in the receptor solution after 24 hours because it is not soluble in the fatty base of this formulation; however, it was detected when delivered from the hydroalcoholic solution reflecting a better solubility.

Table 5. Quantities per unit area of skin surface of the eleven phenolic compounds detected in the receptor solution after 3, 6 and 24 hours.

Compound	Quantity per unit area of skin detected in receptor solution (average ± SD, nmol·cm^{-2}, n=6)					
	50% w/w CP paste			50 mg·mL^{-1} CP solution (ethanol/water; 50:50)		
	3 h	6 h	24 h	3 h	6 h	24 h
Vanillic acid	0.1 ± 0.07	0.5 ± 0.3	4.4 ± 2.9	-	0.4 ± 0.2	8.0 ± 3.7[a]
Verbascoside	-	-	-	-	-	0.2 ± 0.1
4-Coumaric acid	-	-	0.2 ± 0.2	-	-	0.5 ± 0.2
Ferulic acid	-	-	0.3 ± 0.3	-	-	1.1 ± 0.6
Nepetin	0.1 ± 0.02	0.1 ± 0.03	0.4 ± 0.2	-	0.03 ± 0.01	3.2 ± 1.6[b]
Luteolin	-	-	0.05 ± 0.02	-	-	0.3 ± 0.1
Apigenin	-	-	0.1 ± 0.1	-	-	0.9 ± 0.2
Naringenin	-	-	-	-	-	0.01 ± 0.002
Hispidulin	0.2 ± 0.2	0.7 ± 0.5	4.0 ± 2.4	0.1 ± 0.1	1.5 ± 0.8	21.4 ± 3.7[b]
Hesperetin	-	-	0.2 ± 0.1	-	-	0.3 ± 0.1
Chrysin	-	-	-	-	-	-

a = significant difference at $P < 0.05$; b = significant difference at $P < 0.001$ when compare the two formulations at the same sampling time.

Direct comparison of the theoretically predicted J_{max} values in Table 4 with the experimental data in Table 5 is not possible because the degrees of saturation of the different compounds in the two formulations are unknown. However, from a qualitative standpoint, it is perhaps reassuring to observe that verbascoside was expected to penetrate the skin very poorly and this was indeed the case. Equally, vanillic acid was well-absorbed and this was consistent with the relatively high J_{max} predicted from the model; nonetheless, the measured penetration of this compound was well below that anticipated from J_{max} suggesting that vanillic acid was present in the formulations at levels much less than the saturation concentration. The same is almost certainly true for 4-coumaric acid, ferulic acid and chrysin, for which no detectable skin penetration was found. Interestingly, the predicted J_{max} values of nepetin and hispidulin would roughly suggest absorptions of 4.8 and 9.6 nmol·cm^{-2}, respectively, in 24 hours, values not that different from those observed experimentally (and suggesting, therefore, that these constituents were close to saturation in the vehicles).

Various types of bioactivity have been reported for vanillic acid, nepetin and hispidulin, such as antimicrobial (Delaquis *et al.*, 2005; Sultana and Afolayan, 2007), anti-inflammatory (Clavin *et al.*, 2007; Gil *et al.*, 1994; Kim *et al.*, 2011), and antioxidant (Kang *et al.*, 2009), that may be used to predict the potential pharmacological activities of topical CP products in Thai traditional medicine. Interestingly, even though verbascoside is unlikely to penetrate through the skin, it possesses antimicrobial activity against *Straphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Shikanga *et al.*, 2010). Thus, it may at least be beneficial as a topical antimicrobial compound.

4. Conclusions

We have examined the profile of occurrence and skin penetration of polyphenolic compounds of *C. petasites* to evaluate the topical uses of this plant in Thai traditional medicine. Nine phenolic compounds, vanillic acid, 4-coumaric acid, ferulic acid, verbascoside, nepetin, luteolin, chrysin, naringenin, and hesperetin that have not previously been reported from the species, plus apigenin

and hispidulin, were identified in CP extracts. Hispidulin was quantified as a predominant compound, being present at 39 µmol/g in a dried ethanolic extract (equal to 0.04% by weight in dried crude plant material). Using a pig skin model, vanillic acid, nepetin and hispidulin were found to be able to penetrate the skin. Reasonable predicted maximum fluxes and together with biological activities reported elsewhere, of these compounds potentially support the topical clinical uses of this plant in Thai folklore.

The results in this study illustrate that topical bioavailability of individual constituents of herbal preparations may limit which compounds actually act at a pharmacological level, and that this is determined by both the levels in the plant and the ability of the compound to penetrate the skin. In particular, various glycosides, which commonly occur in plant materials and which possess a wide range of potentially useful biological properties, may not be absorbed transdermally. The results reported here show that such compounds are readily identified from their physicochemical properties. However, compounds with modest molecular weight and a balanced lipophilicity (as measured by log P), are both predicted and demonstrated to have reasonable percutaneous absorbability. Taken together, a quantitative analysis of the plant material, the application of the Potts and Guy equation, and knowledge of the *in vitro* biological activities of individual chemical components provide a useful approach to the evaluation of traditional topical herbal preparations and for further formulation optimisation of those products.

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